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The RACK1 Ortholog Asc1 Functions as a G-protein β Subunit Coupled to Glucose Responsiveness in Yeast*

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Corinne E. Zeller, Stephen C. Parnell, and Henrik G. Dohlman[1]

From the Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

According to the prevailing paradigm, G-proteins are composed of three subunits, an α subunit with GTPase activity and a tightly associated βγ subunit complex. In the yeast *Saccharomyces cerevisiae* there are two known Gα proteins (Gpa1 and Gpa2) but only one Gβγ, which binds only to Gpa1. Here we show that the yeast ortholog of RACK1 (receptor for activated protein kinase C1) Asc1 functions as the Gβ for Gpa2. As with other known Gβ proteins, Asc1 has a 7-WD domain structure, interacts directly with the Gα in a guanine nucleotide-dependent manner, and inhibits Gα guanine nucleotide exchange activity. In addition, Asc1 binds to the effector enzyme adenylyl cyclase (Cyr1), and diminishes the production of cAMP in response to glucose stimulation. Thus, whereas Gpa2 promotes glucose signaling through elevated production of cAMP, Asc1 has opposing effects on these same processes. Our findings reveal the existence of an unusual Gβ subunit, one having multiple functions within the cell in addition to serving as a signal transducer for cell surface receptors and intracellular effectors.

All cells must be able to detect extracellular signals and environmental changes, and then mount an appropriate response to those signals. Specific stimuli include light, hormones, neurotransmitters, growth factors, and odorants. Guanine nucleotide-binding proteins (G-proteins)[2] are key intermediates in the cellular signaling process, by coupling cell surface receptors with intracellular effector proteins that generate cellular responses (1, 2).

The prototypical heterotrimeric G-protein consists of α, β, and γ subunits and is coupled to a seven-transmembrane receptor at the plasma membrane. Upon binding of agonist to the receptor, a conformational change in the Gα subunit promotes the release of GDP and binding to GTP. Guanine nucleotide exchange triggers Gβγ disassociation from the Gα subunit, after which both components are free to activate or inhibit downstream effectors. The Gα subunit eventually hydrolyzes GTP to GDP, resulting in re-association of the heterotrimer complex and termination of signaling (2).

The Gβγ complex functions on many levels to promote, and restrict, signaling at the plasma membrane. Most Gβγ complexes help to recruit Gα subunits to the plasma membrane and promote coupling of the G protein to the receptor. The Gβγ also functions as a guanine nucleotide disassociation inhibitor to prevent spontaneous exchange of GTP for GDP by the Gα. Finally, many Gβγ subunits regulate downstream effector enzymes. Gβγ subunits regulate a wide variety of effector enzymes including adenylyl cyclase, which converts ATP to the second messenger cAMP (2–5).

The yeast *Saccharomyces cerevisiae* has two G-protein signaling pathways. The first is the pheromone response pathway, which is among the best characterized stimulus-response pathways of any system. In this example, signaling is activated by secreted peptide pheromones. Cell surface pheromone receptors activate a G-protein heterotrimer, in which the Gβγ (Ste4/Ste18) is released and activates a downstream MAP (mitogen-activated protein) kinase cascade at the plasma membrane (6–8). The Gα subunit (Gpa1) activates a distinct effector, a phosphatidylinositol 3-kinase (Vps34) located at the endosomal membrane (9). These signaling events cooperate to promote mating or fusion of haploid **a** and α cells to form the **a**/α diploid.

A second G-protein signaling pathway mediates pseudohyphal differentiation in diploids and invasive growth in haploids. Both of these responses are mediated by changes in the nutrient status of the growth medium. In diploids, cells transition to pseudohyphal differentiation upon limitation of nitrogen (10); whereas in haploids, the cells transition to invasive growth upon limitation of glucose (11) or other signals that cause an increase in cAMP (12). Both differentiation behaviors are characterized by altered budding, formation of long branching filaments, as well as increased adherence and invasion of the substratum. Collectively, such filamentous growth may help to direct new cells to sites of improved growth conditions.

The same cellular machinery is used for invasive growth in both haploids and diploids, and is minimally comprised of a cell surface receptor (Gpr1), a Gα protein (Gpa2), and the adenylyl cyclase (Cyr1). All of these proteins are required for glucose-induced cAMP signaling (13) and pseudohyphal differentiation (14, 15). There is also evidence for physical association of glucose with Gpr1 (16), Gpr1 with Gpa2 (17–19), and Gpa2 with Cyr1 (20, 21). The resulting increase in cAMP promotes activation of the cAMP-dependent protein kinase (22–27). Cyr1 is also directly activated by the small G protein Ras2 (28), although this activation event occurs independently of Gpr1

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[1] To whom correspondence should be addressed: 405 Mary Ellen Jones Bldg., Campus Box 7260, Chapel Hill, NC 27599-7260. Tel.: 919-843-6894; E-mail: henrik_dohlman@med.unc.edu.

[2] The abbreviations used are: G-protein, guanine nucleotide-binding protein; FRE, filamentous response elements; MAP kinase, mitogen-activated protein kinase; GST, glutathione *S*-transferase; LIC, ligation-independent cloning; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; MES, 4-morpholineethanesulfonic acid; RACK1, receptor for activated protein kinase C1.

and Gpa2 (29). A second glucose-response pathway requires Ras2, but leads to activation of a MAP kinase cascade comprised of Ste20, Ste11, Ste7, and Kss1 (30, 31). The glucose receptor in this second pathway has not been identified, although it does not appear to be Gpr1 and does not require Gpa2 (14, 29, 32, 33).

Based upon sequence and structural comparison of typical G protein subunits within the yeast genome, there appears to be only one canonical Gβγ (Ste4/Ste18). Early research revealed that Ste4 and Ste18 function exclusively in conjunction with Gpa1 in the pheromone-response pathway (32). Two kelch-repeat proteins, Gpb1 and Gbp2 (also known as Krh1 and Krh2), were proposed to function as the Gβ subunits for Gpa2 (34). However, further investigation has shown that these proteins are not Gβ-mimics but instead function to inhibit signaling downstream of Gpa2 (21, 35–37). Using bioinformatic, genomic, biochemical, and pharmacological approaches we have identified Asc1 as the Gβ for Gpa2, and demonstrate a critical role for Asc1 in the glucose signaling pathway mediated by adenylyl cyclase.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Standard methods for the growth, maintenance, and transformation of bacteria and yeast and for the manipulation of DNA were used throughout. Yeast *Saccharomyces cerevisiae* strains used in this study were BY4741 (*MAT***a** *leu2*Δ *met15*Δ *his3*Δ *ura3*Δ) and BY4741-derived gene deletion mutants (Invitrogen), and Σ1278-based invasive strain (*MAT***a** *leu2*Δ *ura3*Δ, from Joseph Heitman, Duke University) and Σ1278-derived gene deletion mutants. Yeast shuttle plasmids pRS315-ADH (CEN, amp^R, *LEU2*, *ADH1* promoter/terminator) and pRS316-ADH (CEN, amp^R, *URA3*, *ADH1* promoter/terminator) (38) were modified by PCR amplification of the gene of interest and subcloned such that a FLAG (GATTACAAGGATGACGACGATAAG) or Myc (GAACAAAAATTGATTTCTGAAGAAGATTTG) epitope could be added to the 5′ coding sequence of any gene with a SacI site engineered in-frame with the open reading frame. Previously described yeast shuttle plasmids used in this study are pAD4M-GST (38) and pAD4M-GPA2-GST (39). The FRE-lacZ transcription reporter plasmid was generously provided by Gerald Fink (40). The *Escherichia coli* expression plasmids were constructed by PCR amplification of the *ASC1* (also known as *CPC2*) and *GPA2* coding regions and annealed into gapped ligation-independent cloning (LIC) vectors pLIC-HIS and/or pLIC-GST (from John Sondek, University of North Carolina).

Purification of Proteins from Yeast—Transformed strains were grown to $A_{600\ nm} \sim 1.0$, harvested by centrifugation, and resuspended in FLAG Lysis Buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 25 mM β-glycerolphosphate, 0.1% Triton-X100, protease inhibitor mixture pellets (Roche, 1 pellet/25 ml of buffer)). Cells were lysed by vortexing with glass beads 10 times, 30 s each, with cooling on ice for 1 min in between. Lysates were rocked at 4 °C for 30 min to solubilize membrane proteins, and centrifuged at 6,000 × *g* for 1 min and again for 30 min to remove insoluble matter. Protein content of the supernatant was determined by DC Protein Assay (Bio-Rad), equalized, and mixed with 20 μl of M2 FLAG Affinity Resin (Sigma) equilibrated with FLAG Lysis Buffer. After 2 h of gentle rocking at 4 °C, the resin was collected by centrifugation at 500 × *g* for 1 min. Resin was washed 4 times with 1.5 ml of FLAG Lysis Buffer and bound proteins were eluted with 75 μl of 2× SDS-PAGE sample buffer at 100 °C for 5 min. For experiments comparing binding between GDP or GDP-AlF_4^- loaded Gα proteins, 10 μM GDP or 10 μM GDP with 30 μM $AlCl_3$ and 10 mM NaF, respectively, was added to the lysis and wash buffers. Samples were resolved by SDS-PAGE and immunoblotting with 9E10 anti-Myc (41) and anti-FLAG monoclonal antibodies (Sigma), in conjunction with enhanced chemiluminescence detection of horseradish peroxidase-conjugated secondary antibodies.

Expression and Purification of Proteins from E. coli—pLIC-HIS-ASC1, pLIC-GST-ASC1, and pLIC-HIS-GPA2 were transformed into *E. coli* strain BL21. Overnight cultures grown at 37 °C from single colonies in Luria Broth containing 100 μg/ml carbenicillin were diluted into fresh medium to $A_{590\ nm} \sim 0.1$ at 37 °C. Once the cultures reached $A_{590\ nm} \sim 0.6$ they were transferred to 25 °C and grown to $A_{590\ nm} \sim 0.9$. Protein expression was induced by addition of 25 mM isopropyl β-D-1-thiogalactopyranoside for an additional 5 h. Cells were harvested by centrifugation for 15 min at 6,000 × *g*. Cells expressing GST fusion proteins were resuspended in GST Lysis Buffer (10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 1 M NaCl, 2.5 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, protease inhibitor mixture pellets). Cells expressing His fusion proteins were resuspended in His Lysis Buffer (50 mM Tris, pH 8.0, 1 M NaCl, 0.1% Triton X-100, 15 mM imidazole, 1 mM dithiothreitol, protease inhibitor mixture pellets) at 4 °C and the cells were lysed using an Emulsiflex-C5 Homogenizer (Avestin Inc.). 50 μg/ml of DNase I was added to the lysates and gently rocked at 4 °C for 30 min followed by centrifugation at 12,000 × *g* for 30 min. GST lysates were mixed with glutathione-Sepharose 4 Fast Flow (GE Healthcare) equilibrated with GST Lysis Buffer; His lysates were mixed with nickel-Sepharose 6 Fast Flow (GE Healthcare) equilibrated in His Lysis Buffer. Lysates were incubated with beads for 2 h at 4 °C with gentle rocking. The beads were then loaded into a chromatography column and washed with appropriate buffer until the flow through contained no protein as detectable by the DC Protein Assay (Bio-Rad). GST fusion proteins were eluted with GST Lysis Buffer supplemented with 10 mM reduced glutathione, and then exchanged into Storage Buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol); His fusion proteins were eluted with His Lysis Buffer supplemented with 250 mM imidazole and then exchanged into Storage Buffer. Elution and Storage Buffers for the purification of His-Asc1 included 25% glycerol, whereas all other buffers contained 5% glycerol. All buffers for the purification of Gpa2 included 10 μM GDP. Purified proteins were concentrated using a Vivaspin (Vivascience) concentrator.

In Vitro Binding Assay—Purified His-Gpa2 (final concentration 200 nM) was reconstituted with 10-fold molar excess GST-Asc1 in 1.0 ml of Binding Buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5% glycerol, 1 mM dithiothreitol, 3 mM $MgCl_2$, 10 μM GDP) with or without 10 mM NaF and 30 μM $AlCl_3$ (AlF_4^-) and gently mixed for 30 min at 4 °C. 50 μl of Glutathione-Sepharose

4 was equilibrated with Binding Buffer and then mixed with the protein samples for 2 h at 4 °C with gentle rocking. Beads were collected by centrifugation at 500 × *g* for 1 min and washed 4 times with Binding Buffer. Bound proteins were eluted with 50 μl of 2× SDS-PAGE buffer at 100 °C for 5 min and resolved by SDS-PAGE and immunoblotting with anti-GST (from Joan Steitz, Yale University) or anti-His_6 (Qiagen) antibodies.

Invasive Growth Assay—5 μl of an overnight saturated culture of the indicated strains from the Σ1278 background were spotted onto a YPD plate and grown at 30 °C for 3 days. Total growth of strains was imaged, and then the plate was gently washed under a stream of water to remove non-invasive cells from the surface of the agar. The plates were allowed to dry briefly before re-imaging to document invasive growth.

GTPγS Binding Assay—200 nM His-Gpa2 was preincubated alone or with either 200 nM or 1 μM His-Asc1 in BODIPY Buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 10 mM $MgCl_2$) for 30 min at 27 °C. Measurement of BODIPY FL-GTPγS (Molecular Probes, Inc.) binding to His-Gpa2 was determined as described previously (42).

Steady-state GTP Hydrolysis Assay—200 nM His-Gpa2 was preincubated alone or with 200 nM, 1 μM, or 4 μM His-Asc1 in Hydrolysis Buffer (50 mM HEPES, pH 8.0, 1 mM EDTA, 3 mM dithiothreitol, 0.025% Lubrol) for 30 min. The rate of GTP hydrolysis was determined as described previously (43).

Transcription Reporter Assay—Strains were transformed with the FRE-lacZ reporter plasmid, grown in selective SCD-LEU medium overnight, and then diluted to $A_{600\,nm} = 0.2$ in fresh medium and grown for an additional 20 h at 30 °C. Cells were collected by centrifugation and washed twice with sterile water and once with MES Buffer (10 mM MES, pH 8.0, 0.1 mM EDTA) and finally resuspended in MES Buffer and incubated for 2 h at 30 °C with shaking. Cultures were diluted to equalize density, and 90 μl of cells were mixed with 10 μl of D- or L-glucose at 0–100 mM final concentration, and incubated for 90 min at 30 °C. β-Galactosidase activity was measured as described previously (44).

cAMP Production Assay— cAMP was measured using a cAMP Biotrak EIA kit (GE Healthcare) as described previously (15).

MAP Kinase Phosphorylation Assay—Overnight cultures grown in YPD-rich medium were diluted to $A_{600\,nm} = 0.2$ in fresh YPD and grown for an additional 20 h at 30 °C. Cells were collected by centrifugation and washed twice with sterile water and once with MES Buffer and finally resuspended in MES Buffer and incubated for 2 h at 30 °C with shaking. After 2 h the cultures were treated with 100 mM D- or L-glucose and 750 μl of culture was collected at the indicated times by rapidly mixing with 750 μl of 2× trichloroacetic acid Lysis Buffer (20 mM Tris-HCl, pH 8.0, 20% trichloroacetic acid, 50 mM NH_4OAc, 2 mM Na_2EDTA) and flash frozen in liquid nitrogen. Protein was extracted as described previously (45) and quantified using the DC protein assay (Bio-Rad). A total of 50 μg of protein extracts was resolved by 12.5% SDS-PAGE and immunoblotting with p44/42 MAPK antibodies (Cell Signaling Technology) (46).

RESULTS

The 7-WD Repeat Protein Asc1 Interacts Directly with the Inactive Form of Gα Gpa2—Our objective in this study was to identify the Gβ protein for Gpa2. The crystal structures of the mammalian Gβγ subunits of transducin revealed that the Gβ protein folds into a seven-bladed propeller structure (β-propeller) based around 7-WD repeat motifs (47). The WD repeat is a conserved domain, 40–60 amino acids in length that begins with glycine-histidine and ends with tryptophan-aspartic acid (WD). Because all previously identified Gβ subunits have the characteristic 7-WD domain structure, we began by searching for any gene product bearing this signature motif. We did not limit our search to proteins of unknown function, because WD repeat-containing proteins often have broad or even multiple functions, including roles in signal transduction, cell cycle control, mRNA splicing, and transcriptional repression (48). In yeast alone, there are an estimated 104 proteins that contain WD repeats (49). By searching the SMART (Simple Modular Architecture Research Tool) data base (49) we identified 21 proteins predicted to have either seven WD repeats, or in some cases six repeats with an additional segment large enough to contain a seventh (less-conserved) WD domain.

We then considered which of the 21 candidate proteins could function in the manner of known Gβ proteins. In addition to their characteristic 7-WD structure, Gβ proteins (i) bind directly to Gα subunits; (ii) bind preferentially to the inactive GDP-bound form of the Gα protein; (iii) function as guanine nucleotide disassociation inhibitors; and, in most cases (iv) regulate downstream signaling events (3). We initially searched for proteins able to bind to Gpa2 *in vivo*. All 21 candidates were fused to the FLAG epitope and individually expressed in yeast together with Gpa2 fused to GST. Each of the FLAG fusion proteins was then immunoprecipitated. The presence of Gpa2 in the resulting immunoprecipitates was detected by SDS-PAGE and immunoblotting using anti-GST antibodies. This analysis identified two proteins capable of co-precipitating Gpa2: Asc1 and Prp4 (data not shown).

We next sought to determine whether either Asc1 or Prp4 binds preferentially to the inactive (GDP bound) form of Gpa2. Asc1 and Prp4 were again immunoprecipitated, this time in the absence or presence of AlF_4^-. GDP-AlF_4^- mimics the transition state for GTP hydrolysis, and induces a conformation resembling that of the activated Gα protein (2). In this comparison, Prp4 bound equally to both the active and inactive forms of Gpa2, and was eliminated from further consideration. In contrast, Asc1 bound almost 4-fold more Gpa2-GDP than Gpa2-GDP-AlF_4^- (Fig. 1*A*). Thus Asc1 binds preferentially to the inactive form of Gpa2, in the manner of known Gβ-Gα interactions.

Having shown that Asc1 binds preferentially to inactive Gpa2, we sought to determine whether the interaction is direct. To this end we purified GST-Asc1 and His_6-Gpa2 from *E. coli*, and reconstituted the proteins in the presence of GDP or GDP-AlF_4^-. Asc1 was then re-purified by glutathione affinity chromatography, and any co-purifying Gpa2 was detected by immu-

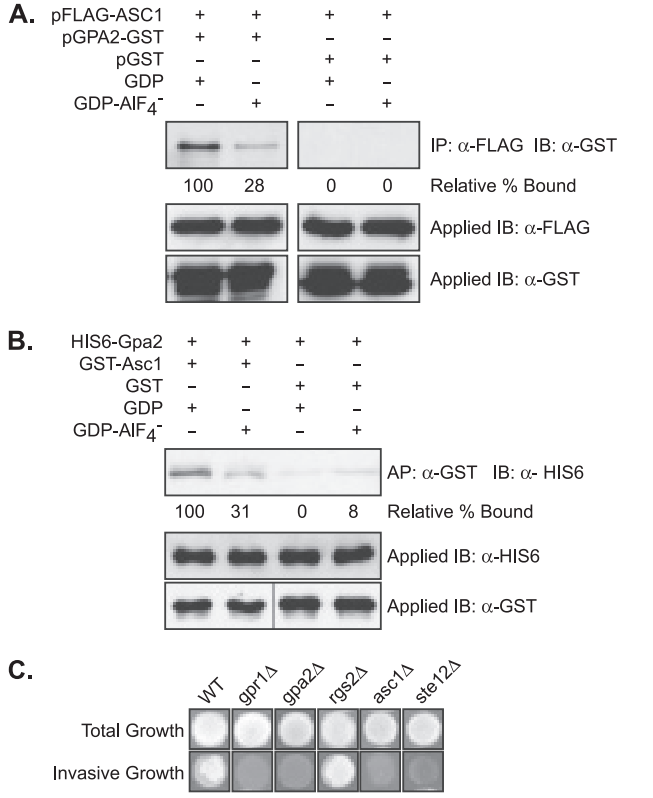

FIGURE 1. **Asc1 interacts with the inactive GDP-bound form of the Gα Gpa2 and is required for invasive growth.** *A*, co-immunoprecipitation of Gpa2 with Asc1 from yeast. Whole cell extracts from wild-type cells transformed with plasmids containing FLAG-ASC1 (pRS316-ADH) and either GPA2-GST or GST alone (pAD4M) were immunoprecipitated (*IP*) in buffer containing either GDP or GDP-AlF_4^- with anti-FLAG beads, washed, eluted with SDS-PAGE sample buffer, and resolved by SDS-PAGE and immunoblotting (*IB*). Co-purifying proteins were detected with anti-GST antibodies. Relative binding (% of maximum) was determined by densitometry. Applied samples are 10% (50 ng) of the starting protein sample used for precipitation. *B*, co-purification of Gpa2 with Asc1 using recombinant proteins purified from *E. coli*. 0.2 nmol of Gpa2 was preincubated with 2.0 nmol of Asc1 for 30 min and then repurified by GST-Sepharose affinity chromatography. Samples were eluted and resolved as described above, except that Gpa2 was detected with anti-His_6 antibodies. *C*, invasive growth assay. Cells containing the indicated deletions from the Σ1278-based strain were spotted onto YPD plates and grown for 3 days at 30 °C (total growth) and then gently washed to detect invasive growth.

noblotting with anti-His_6 antibodies. As shown in Fig. 1*B*, Asc1 binds directly to Gpa2, and binds preferentially to the inactive (GDP-bound) form of the protein.

Disruption of Gpr1 or Gpa2 activity has been reported to impair specific growth behavior phenotypes, including diploid pseudohyphal and haploid invasive growth (14, 15). As we have shown that Asc1 interacts directly with Gpa2 we sought to establish whether *ASC1*-deficient strains have a phenotype in a Gpa2 signaling pathway, and to rule out the possibility of functional redundancy with another Gβ-like protein. Therefore, we examined the ability of *asc1*Δ cells to sustain the invasive growth response. Fig. 1*C* shows that *ASC1* is required for invasive growth. As has been shown previously, *GPR1* (15), *GPA2* (37), and *STE12* (50) are likewise required for invasive growth. In contrast, the wild-type and *rgs2*Δ strains are invasive. These results show that Asc1 both interacts with Gpa2 and is important in Gpa2-mediated signaling.

Asc1 Functions as a Guanine Nucleotide Dissociation Inhibitor—Having shown that Asc1 binds, in the manner of known Gβ subunits, to Gpa2, we next sought to characterize Asc1 biochemically. Gβ proteins inhibit the spontaneous exchange of GTP for GDP on their cognate Gα. To determine whether Asc1 has the same function toward Gpa2, we measured the rates of GTP binding and hydrolysis. In the absence of receptors, the rate-limiting step in the GTPase cycle is the release of GDP (2, 51); thus if Asc1 reduces the spontaneous rate of GDP release, the rates of GTP binding and hydrolysis should be correspondingly diminished.

We first determined if Asc1 affects the rate of spontaneous GTP binding to Gpa2. For these experiments we used a fluorescent non-hydrolyzable analogue of GTP (BODIPY-GTPγS). The fluorescence of this ligand is quenched in solution but increases dramatically upon binding to Gα. Therefore, the rate of GTP binding can be easily quantified by fluorometry (52). Purified recombinant His_6-Gpa2, either alone or in the presence of a 5-fold molar excess of purified recombinant His_6-Asc1, was mixed with BODIPY-GTPγS and the resulting increase in fluorescence was monitored over time. The initial rate of nucleotide exchange (as measured by relative fluorescence per second for the first 60 s) for Gpa2 alone was 0.096 ± 0.006 relative fluorescence units/s, and in the presence of Asc1 the rate was reduced to 0.061 ± 0.003 relative fluorescence units/s (Fig. 2*A*).

We then measured GTP hydrolysis under steady state conditions. For this experiment we used purified Gpa2 (final concentration 200 nM), either alone or with Asc1 in equimolar, 5-fold excess, or 20-fold excess concentrations. The proteins were incubated with [γ-^{32}P]GTP, and the amount of ^{32}P released was determined over time. As shown in Fig. 2*B*, increasing concentrations of Asc1 yielded a corresponding decrease in the rate of [γ-^{32}P]GTP hydrolysis, from 0.78 (no Asc1) to 0.42 pmol/min (equimolar Asc1), 0.34 pmol/min (5-fold excess of Asc1), and 0.26 pmol/min (20-fold excess of Asc1). Based on the results of the binding and hydrolysis assays, we conclude that Asc1 functions biochemically to inhibit Gpa2 guanine nucleotide exchange, in the manner of known Gβ proteins.

Asc1 Negatively Regulates Glucose-mediated Signaling—Having determined that Asc1 binds to Gpa2, has a phenotype in Gpa2 mediated signaling, and regulates the biochemical activity of Gpa2, we next sought to determine the physiological role of Asc1 in glucose signaling. Two signaling processes, linked through Ras2, have been reported to be activated by extracellular glucose. In the first case, glucose binds to an unknown receptor and promotes activation of a MAP kinase cascade that includes Ste20, Ste11, Ste7, and Kss1 (30, 31). In the second case, glucose binds to the receptor Gpr1 (16), which activates the Gα protein Gpa2, which in turn activates the adenylyl cyclase Cyr1 (13–27, 53, 54). Activation of Cyr1 leads to a rapid and transient increase in cellular concentrations of cAMP (19).

We first investigated the glucose-response pathway leading to Kss1 MAP kinase activation. It is not known how glucose activates this particular pathway, but it appears to terminate with transcription factors that bind filamentous

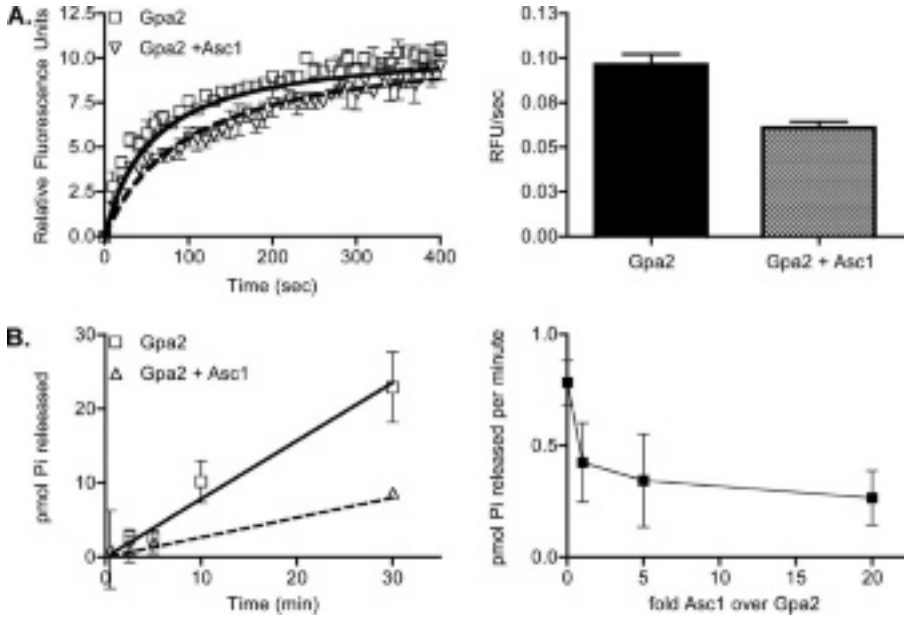

FIGURE 2. **Asc1 functions as a guanine nucleotide dissociation inhibitor.** *In vitro* GTP binding assays using recombinant protein purified from *E. coli*. *A*, 200 nmol of Gpa2 was preincubated either alone or with 1000 nmol of Asc1 for 30 min. Rate of BODIPY-GTPγS binding to Gpa2 was then determined as the increase in fluorescence (*left panel*). Initial rates are 0.093 relative fluorescence units (*RFU*)/s for Gpa2 and 0.061 relative fluorescence units/s for Gpa2 plus Asc1 (*right panel*). *B*, steady-state GTP hydrolysis assay. 200 nmol of Gpa2 was preincubated either alone or with 200, 1000, or 4000 nmol of Asc1 for 30 min. Rate of hydrolysis was determined by measuring the release of free ^{32}P over time. A representative time course is shown for Gpa2 alone and Gpa2 with 4000 nmol of Asc1 (*left panel*). Rate of hydrolysis shown for all conditions is shown in the *right panel*. All data are representative of two or more separate experiments. *Error bars* ± S.E.

response elements (FREs) (30, 31, 55). To determine whether Asc1 affects glucose-mediated signaling through Kss1 we measured gene transcription activity using an FRE(*TEC1*)-lacZ reporter (40, 56). This reporter responds to activation by the transcription factor heterodimer composed of Ste12 and Tec1, which is in turn activated by Kss1 (40, 57–59). In this method cells must first be grown to saturation and then further starved in nutrient-free medium to impose glucose exhaustion. Wild-type as well as *gpr1*Δ, *gpa2*Δ, *ste20*Δ, and *asc1*Δ mutant strains were grown to saturation, starved, and then stimulated with D-glucose for 45 min. Induction of lacZ (β-galactosidase) was determined spectrophotometrically. Compared with wild-type cells, the *asc1*Δ strain is greatly more sensitive to glucose (EC_{50} = 0.95 *versus* 8.08 mM, Fig. 3*A*). Moreover, the *asc1*Δ strain also exhibited substantially elevated basal and maximal transcription activity. The *gpr1*Δ and *gpa2*Δ strains had diminished responses and the *ste20*Δ strain exhibited no response to glucose (Fig. 3*B*). The phenotype of the *asc1*Δ strain was rescued by transforming cells with a plasmid containing a genomic copy of *ASC1* (data not shown). Yeast are only able to utilize the D-enantiomer of glucose (16); as expected, cells treated with L-glucose exhibited no β-galactosidase induction (data not shown). These results indicate that Asc1 represses new gene transcription under basal as well as glucose-stimulated conditions; and that Gpr1 and Gpa2 do not significantly contribute to glucose signaling through the MAP kinase cascade. This result is in agreement with previous studies showing that Gpr1 and Gpa2 are not required to activate Kss1 (60).

In the pheromone response pathway, the Gβ protein Ste4 binds to Ste20, and this binding is required for Fus3 MAP kinase activation (61). Therefore, we asked if the Gβ protein Asc1 has a similar ability to bind Ste20, and perhaps in this way serves to regulate Kss1. To test this hypothesis, FLAG-tagged Ste20 was co-expressed with Myc-tagged Asc1 in yeast and Ste20 was purified using an anti-FLAG affinity resin. As shown in Fig. 3*C*, Asc1 copurifies with Ste20. To determine whether Asc1 interaction is required for glucose signaling, we determined the physiological consequences of disrupting this interaction. We compared the glucose activation of Kss1 in a wild-type strain and isogenic mutant strains lacking components of the G-protein signaling apparatus including *ASC1*. Kss1 kinase activation was monitored using phospho-p42/p44 antibodies, which recognize the dually phosphorylated and activated forms of both Kss1 and Fus3 (46). As shown in Fig. 3*D*, glucose stimulation of wild-type cells led to Kss1 activation within 8 min, and maximal induction by 12 to 16 min. The *asc1*Δ strain showed a similar activation trend, however, there was a much higher level of basal as well as maximal Kss1 phosphorylation. The *gpr1*Δ and *gpa2*Δ strains showed similar responses to wild-type (Fig. 3*D*). Again, the *asc1*Δ signaling phenotype was rescued by transformation of the cells with a plasmid containing a genomic copy of *ASC1* (data not shown).

These results indicate that Asc1 interacts with Ste20 and functions to repress basal MAP kinase activity, without substantially altering MAP kinase activation in response to glucose stimulation. However, this is in contrast to Ste4, where binding to Ste20 leads to activation of MAP kinase signaling (61). We hypothesize that the diminished transcriptional response seen in the *gpr1*Δ and *gpa2*Δ strains is caused by release of Asc1, allowing it to interact instead with Ste20 and thereby inhibit MAP kinase signaling.

We then considered a role for Asc1 in the second branch of the glucose signaling pathway, in this case leading to activation of adenylyl cyclase. In mammalian cells, it is well established that Gα and Gβγ proteins can act on the same effector enzyme including most isoforms of adenylyl cyclases. For example, type I adenylyl cyclase is activated by $G\alpha_s$ and inhibited by Gβγ, whereas type II adenylyl cyclase is activated by both $G\alpha_s$ and Gβγ (5). The yeast adenylyl cyclase is activated in response to glucose stimulation, and this process requires the glucose receptor Gpr1 and the Gα subunit Gpa2 (13–27, 53, 54). Given that many Gβ proteins interact with adenylyl cyclases (3, 5), we investigated whether Asc1 also

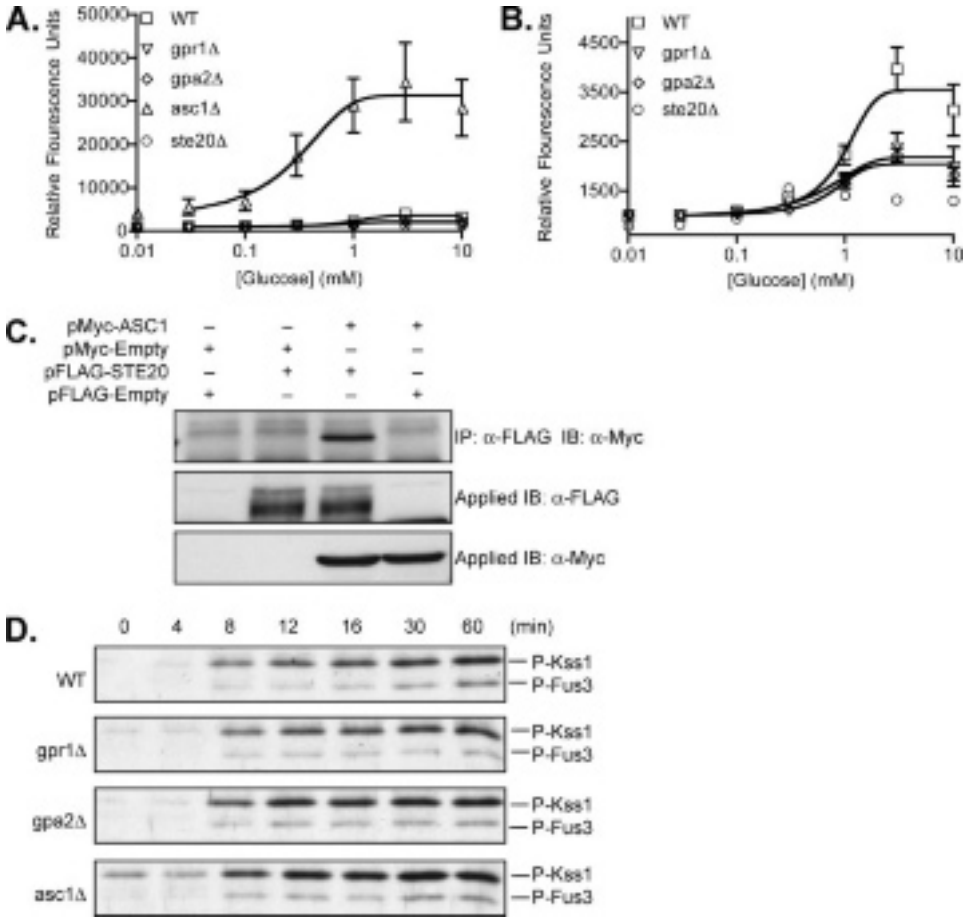

FIGURE 3. **Asc1 interacts with Ste20 and negatively regulates basal signaling of the MAP kinase branch of the glucose signaling pathway.** *A*, D-glucose-dependent transcriptional induction (FRE(*TEC1*) promoter, lacZ reporter) was measured in wild-type, *gpr1*Δ, *gpa2*Δ, *ste20*Δ, and *asc1*Δ cells. Cells were treated with the indicated concentrations of glucose for 60 min and the resulting β-galactosidase activity was measured spectrofluorometrically. The data shown are representative of two independent experiments performed in triplicate. *Error bars* ± S.E. *B*, same graph as in *A* with the *y* axis expanded to the scale of the maximum wild-type response. *C*, Asc1 interacts with Ste20. *gpa2*Δ cells transformed with the indicated plasmids were grown to mid-log phase, lysed, and the detergent-solubilized protein was immobilized on FLAG beads, washed, and eluted with SDS-PAGE sample buffer. Bound proteins were detected by immunoblotting (*IB*) with antibodies against the Myc or FLAG epitopes. 5% (25 ng) of the soluble cell lysate was probed with antibodies against FLAG or Myc to confirm equivalent levels of protein expression. *D*, glucose-dependent Kss1 activation. BY4741 wild-type, *gpr1*Δ, *gpa2*Δ, and *asc1*Δ cells were grown to saturation, washed, and further starved for 2 h. At the time points indicated, samples were removed and flash frozen in liquid nitrogen. Proteins were extracted with trichloroacetic acid and 50 ng of protein was resolved by SDS-PAGE and immunoblotting against a phospho-specific p44/42 antibody. *IP*, immunoprecipitation.

Taken together, these results indicate that Asc1 is a Gβ protein that links the glucose receptor Gpr1 to the intracellular effector Cyr1. Asc1 also interacts with Ste20 to repress basal MAP kinase signaling, but this interaction has relatively modest effects on the glucose response pathway, mainly in dampening the basal activation state of the pathway.

DISCUSSION

Gpa2 was first identified in 1988 (62) and the yeast genome was fully sequenced by 1996 (63), but as yet no cognate Gβγ has been identified. We have now identified Asc1 as the Gβ protein for Gpa2. Asc1 shares many of the characteristics of known Gβ proteins. Structurally, Asc1 contains 7 WD repeats. Functionally, Asc1 interacts directly with the GDP-bound form of Gpa2, inhibits Gpa2 guanine nucleotide exchange, and regulates a known second messenger in the glucose-response pathway.

Asc1 also exhibits some important structural and functional *differences* with known Gβ subunits. With respect to structure, Asc1 lacks a coiled-coil domain normally found at the N terminus of Gβ proteins. This N-terminal region represents an important determinant for binding to Gγ subunits (2). Thus we speculate that Asc1 does not associate with any Gγ-like protein. In support of this concept, Asc1 is a cytoplasmic protein, whereas Gγ subunits are generally responsible for targeting Gβ and Gα specifically to the plasma membrane (more specifically, prenylation of Gγ anchors the Gβγ dimer to the membrane) (3). Moreover, Asc1 can be stably expressed in *E. coli* despite the absence of any Gγ binding partner. Other Gβ proteins must assemble with a Gγ protein to be stably expressed or purified (2).

Asc1 also has several unique functional characteristics. Asc1 was first identified as a transcription suppressor that associates with the 40 S ribosomal subunit (64). Asc1 has also been shown to be an integral component of the ribosome, and is required for some protein-protein interactions within the ribosome complex (65). In a large-scale systematic screen of the yeast proteome, Asc1 was identified in numerous separate protein complexes having a broad range of functions, including membrane biogenesis and function, protein synthesis and turnover, RNA metabolism and transcription, DNA maintenance, and chro-

interacts with Cyr1. Myc-tagged Asc1 was co-expressed with FLAG-tagged Cyr1, and Cyr1 was purified using an anti-FLAG affinity resin. Co-purifying Asc1 was then identified by immunoblotting with anti-Myc antibodies. As shown in Fig. 4*A*, Asc1 binds to Cyr1, and binding occurs even in the absence of Gpa2 expression (*i.e.* the complex was still detected in *gpa2*Δ mutant cells). We then determined the effect of this interaction on cAMP production. Wild-type as well as *gpr1*Δ and *asc1*Δ mutant yeast strains were grown to saturation, starved for 2 h, and treated with glucose. As shown in Fig. 4*B*, addition of glucose to wild-type cells caused a rapid and transient increase in cAMP, and this response was largely absent in the *gpr1*Δ mutant. In stark contrast, the *asc1*Δ strain exhibited a markedly elevated response, demonstrating that Asc1 inhibits Cyr1 cAMP production.

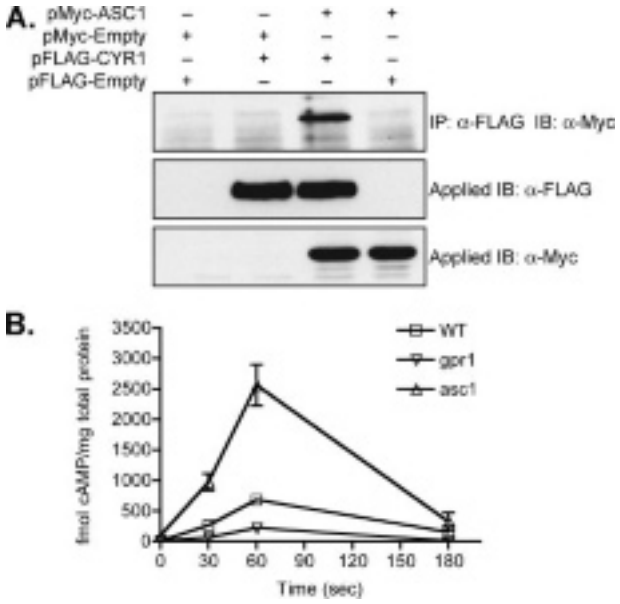

FIGURE 4. **Asc1 interacts with the adenylyl cyclase Cyr1 and negatively regulates the cAMP/PKA branch of the glucose signaling pathway.** *A*, Asc1 interacts with Cyr1. *gpa2*Δ cells transformed with the indicated plasmids were grown to mid-log phase, lysed, and the detergent-solubilized protein was immobilized on FLAG beads, washed, and eluted with SDS-PAGE sample buffer. Bound proteins were detected by immunoblotting (*IB*) with antibodies against the Myc or FLAG epitopes. 5% (25 ng) of the total soluble cell lysate was probed with antibodies against FLAG and Myc to confirm equivalent levels of protein expression. *B*, glucose-dependent cAMP production. Wild-type, *gpr1*Δ, and *asc1*Δ cells were grown to saturation, washed, and further starved for 2 h. Yeast were stimulated with 100 mM glucose and production of cAMP was determined at each time point. *Error bars* ± S.E. *IP*, immunoprecipitation.

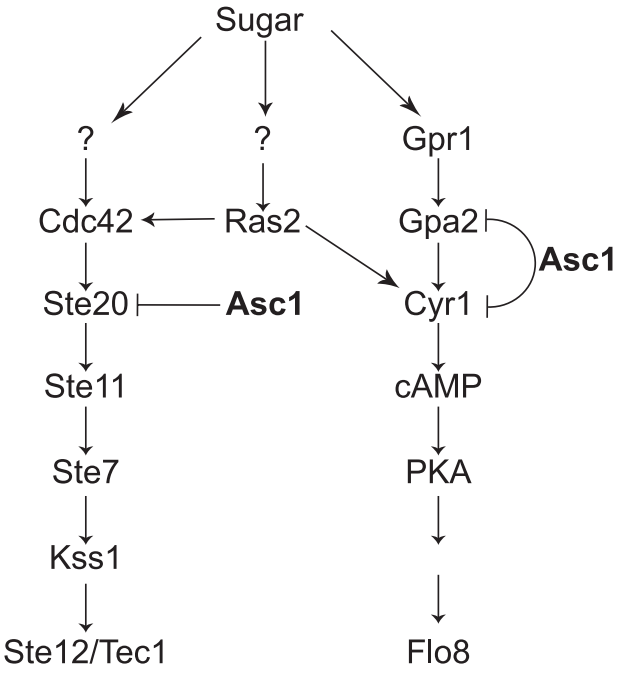

FIGURE 5. **Model of glucose signaling by the MAP kinase and cAMP/PKA signaling pathways.** Under basal conditions, Asc1 functions to (i) inhibit signaling through the MAP kinase module possibly through its interaction with Ste20; and (ii) to inhibit signaling through the cAMP/PKA module by acting as a nucleotide disassociation inhibitor toward Gpa2. Under glucose stimulation, Asc1 functions to (i) inhibit signaling through the MAP kinase module, and (ii) inhibit production of cAMP by Cyr1.

matin structure (66, 67). We have also shown previously that Asc1 interacts weakly with the Gα in the pheromone response pathway, Gpa1, and deletion of *ASC1* results in cells that are slightly more sensitive to pheromone stimulation (39). Such diversity of function has no precedent among the more "typical" Gβ subunits. Notably, Asc1 is a very highly expressed protein with an estimated 330,000 copies per cell (68, 69). This represents a >70-fold excess over Gpa2, with an estimated 4570 copies per cell (68). This difference likely reflects the broad array of functions ascribed to Asc1, most of which are likely to be independent of Gpa2.

Asc1 is closely related to the mammalian protein RACK1, which like Asc1 has diverse functions and is expressed at high levels. RACK1 was first identified as a adaptor for protein kinase C, and was proposed to function in signal transduction (70). RACK1 has since been shown to interact with the G-protein transducin, to repress gene expression as a component of the 40 S ribosome, and to regulate G_1/S progression by suppressing Src kinase activity (71–75). RACK1 and Asc1 have also been shown to be functionally interchangeable within the yeast 40 S ribosome (76).

Asc1 joins a small but growing list of multifunctional or atypical Gβ proteins (77). Recently Gpa1 was shown to bind directly to Vps15 in a GDP-dependent manner. Vps15 is a large protein that contains a protein kinase domain near its N terminus and a 7-WD domain near the C terminus. The 7-WD domain is predicted to fold into a β-propeller structure, in a similar manner to that of Gβ proteins (9). Vps15 also binds to the phosphatidylinositol 3-kinase Vps34, and this binding is required for Vps34 catalytic activity (9). The Asc1 ortholog in *Cryptococcus neoformans*, Gib2, has been identified as an atypical Gβ that functions as a positive regulator of cAMP signaling to regulate melanization and capsule formation associated with virulence (78). These findings are in contrast to our findings that Asc1 is a negative regulator of signaling via cAMP. That study also identified two atypical Gγ subunits, Gpg1 and Gpg2. It is not known, however, if Gib2 has other functions typically ascribed to Gβ subunits, such as receptor coupling, inhibition of guanine-nucleotide exchange, or effector modulation. A third atypical Gβ subunit has recently been described in *Schizosaccharomyces pombe*. This protein, Gnr1, interacts with the Gα from the pheromone response pathway and negatively regulates pheromone signaling (79). Gnr1 functions similarly to Asc1 in that it negatively regulates signaling but is not required to initiate the signaling event.

In summary, we have identified Asc1 as the Gβ subunit in the yeast glucose signaling pathway (Fig. 5). Asc1 binds directly to GDP-Gpa2 and inhibits Gpa2 guanine nucleotide exchange activity. Asc1 also binds to the downstream effector Cyr1, and does so independently of Gpa2 expression. Interaction with Cyr1 appears to result in diminished cAMP production. Asc1 also interacts with a second downstream effector Ste20 and the result of this interaction is repression of basal signaling via the MAP kinase branch of the glucose signaling apparatus.

There is an apparent contradiction between the Gpa2-mediated growth (Fig. 1*C*) and upstream signaling phenotypes (Figs. 3, *A* and *D*, and 4*B*). Whereas *ASC1* is required for invasive growth, as is *GPA2*, the MAP kinase and cAMP signaling phe-

notypes for Gpa2 and Asc1 are opposite: Asc1 restricts these signaling events whereas, Gpa2 promotes them. We speculate that this is due to the *asc1*Δ strain being over-responsive to the presence of glucose. The invasive response is caused by a limitation of glucose; however, in strains lacking *ASC1* the signaling is amplified such that the cells do not detect the glucose limitation and therefore do not become invasive. A second explanation is that a non-Gβ function of Asc1 could be required for invasive growth, but not for glucose-stimulated signaling.

Having identified Asc1 as the Gβ for Gpa2, we are now well positioned to determine how nutritional signals are detected, and how those signals are transduced into changes in cell homeostasis. More generally, the recent discovery of multifunctional Gβ proteins, including Vps15 and Asc1 in yeast, suggests that the superfamily of Gβ subunits may be far larger and more complex than previously recognized.

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